SUPPLEMENTATION OF NITROGEN SOURCES AND GROWTH FACTORS IN PINEAPPLE WASTE EXTRACT MEDIUM FOR OPTIMUM YEAST (*Candida utilis*) BIOMASS PRODUCTION

by

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LIST OF ABBREVIATIONS

Nomenclature

C_L	Dissolved oxygen concentration in the bulk liquid phase (mg/L)
C_S	Dissolved oxygen saturation concentration (mg/L)
dC_L/dt	Dissolved oxygen time derivative (mg/Lh)
DO	Dissolved oxygen (mg/L)
K_La	Oxygen transfer coefficient (h^{-1})
K_S	Monod saturation constant (g/L)
OTR	Oxygen transfer rate (mg/Lh)
OUR	Oxygen uptake rate (mg/Lh)
P	Productivity
pO_2	Dissolved oxygen tension (%)
qO_2	Specific oxygen uptake rate (mg O_2/g cell h)
S	Substrate concentration (g/L)
S_0	Initial substrate concentration (g/L)
S_c	Substrate consumption (%)
t	Time (h)
t_D	Doubling time (h)
x	Biomass concentration (g/L)
x_0	Initial biomass concentration (g/L)
$Y_{X/S}$	Cell mass yield factor (g cell/g S)
μ	Specific growth rate (h^{-1})
μ_{max}	Maximum specific growth rate (h^{-1})

SUPPLEMENTASI SUMBER NITROGEN DAN FAKTOR PERTUMBUHAN KE DALAM MEDIUM EKSTRAK SISA NANAS UNTUK PENGHASILAN OPTIMUM BIOJISIM YIS (*Candida utilis*)

ABSTRAK

Pelbagai sumber nitrogen and faktor pertumbuhan masing-masing ditambah ke dalam medium daripada jus ekstrak daripada sisa nanas (PWE) dan kesan terhadap penghasilan biojisim, hasil dan produktiviti *C. utilis* telah dikaji. Eksperimen dijalankan dengan kelalang kon mengandungi PWE pada 3 ^{o}Brix, pH permulaan pada 4.5 dan inokulum 7.8% v/v (10^6 sel/mL), dieram pada 30 °C dengan kelajuan penggoncangan 100 rpm. Peningkatan signifikan ($p<0.05$) dalam penghasilan biojisim telah diperhatikan apabila penambahan sumber nitrogen tunggal (0.09% nitrogen total, b/i) ditambahkan dengan mengikut turutan berikut: ekstrak yis C1 = $NH_4H_2PO_4$ > pepton ≥ KNO_3 ≥ ekstrak yis C2 = $(NH_4)_2SO_4$. Penambahan gabungan C1 dan $NH_4H_2PO_4$ tidak menunjukkan peningkatan ($p>0.05$) dalam penghasilan biojisim tetapi produktiviti sebanyak 1.84 $gL^{-1}h^{-1}$ direkodkan apabila PWE ditambah dengan 1:1 (asas nitrogen) C1 dan $NH_4H_2PO_4$. PWE bersupplementasi dengan 300µg/L tiamina atau 100 µg/L piridoksina meningkatkan pertumbuhan yis ($p<0.01$). Kaedah sambutan permukaan dijalankan untuk mengkaji interaksi antara sumber-sumber nitrogen and faktor pertumbuhan dengan menggunakan reka bentuk *central composite*. Kepekatan optimum faktor-faktor tersebut terhadap penghasilan biojisim yang maksimum juga ditentukan. Penemuan menunjukkan bahawa penghasilan biojisim adalah dipengaruhi oleh komposisi media pertumbuhan secara signifikan yang mana $NH_4H_2PO_4$ dan tiamina merupakan faktor yang paling mempengaruhi ($p<0.05$). Kepekatan optimum bagi C1 dan $NH_4H_2PO_4$ adalah 0.05% dan 0.10% masing-masing dan ini adalah bersamaan kepada 4.4 g/L dan 8.2 g/L. Kepekatan optimum bagi tiamina dan piridoksina adalah 325 µg/L dan 250 µg/L masing-masing. Keadaan fermentasi yang optimum menghasilkan biojisim

sebanyak 7.57 g/L dengan hasilnya 0.48 g biojisim per g gula penurun yang digunakan.

Kinetik pertumbuhan berkelompok *C. utilis* dalam fermenter 2-L megguna medium PWE, PWE teroptimum dan medium YEPG telah dikaji. Medium PWE teroptimum telah meningkatkan penghasilan biojisim secara signifikan (11.73 g/L) berbanding dengan PWE (8.57 g/L) merupakan akibat peningkatan kadar pertumbuhan spesifik (0.96 h^{-1}). Pemalar penepuan (*Ks*) dan masa mengganda (t_D) telah dikurangkan dengan supplementasi manakala produktiviti (*P*) dan hasil kepada substrates ($Y_{x/s}$) telah diperbaiki serentak. Tetapi, keperluan oksigen (*SOUR*) oleh *C. utilis* dalam medium PWE teroptimum meningkat secara mendadak dan oksigen menjadi faktor penghad pertumbuhan. Kebolehan *C. utilis* untuk menggunakan sukrosa dalam medium PWE selepas jangka masa fermentasi tertentu telah digambarkan melalui mikrograf elektron yang menunjukkan pemendapan retikulum endoplasma sepanjang pinggir membran sel yang menunjukkan sintesis enzim yang mana tidak diperhatikan dalam media YEPG.

SUPPLEMENTATION OF NITROGEN SOURCES AND GROWTH FACTORS IN PINEAPPLE WASTE EXTRACT MEDIUM FOR OPTIMUM YEAST (*Candida utilis)* BIOMASS PRODUCTION

ABSTRACT

Various nitrogen sources and growth factors were respectively incorporated into the growth medium from juice extracted from pineapple waste (PWE) and their effects on *C. utilis* biomass production, yield and productivity were studied. Experiments were conducted in conical flasks containing PWE medium at 3 ^{o}Brix, initial pH of 4.5 and inoculum of 7.8% v/v (10^6 cells/mL), incubated at 30 °C with shaking of 100 rpm. Significant increase ($p<0.05$) in biomass was observed when a single nitrogen source (0.09% total nitrogen, w/v) was added with the following order; yeast extract C1 = $NH_4H_2PO_4$ > peptone ≥ KNO_3 ≥ yeast extract C2 = $(NH_4)_2SO_4$. Addition of combined C1 and $NH_4H_2PO_4$ with the absence of additional growth factor, did not increase ($p>0.05$) the biomass production but the productivity of 1.84 $gL^{-1}h^{-1}$ was recorded when PWE medium was added with 1:1 (N-based) C1 and $NH_4H_2PO_4$. Nitrogen-supplemented PWE medium added with 300 µg/L thiamine or 100 µg/L pyridoxine enhanced the yeast growth ($p<0.01$). Response surface methodology (RSM) was conducted in order to study the interactions between nitrogen sources and growth factors by using central composite design (CCD). Optimum concentrations of these factors on maximum biomass were determined as well. Findings indicated that biomass was significantly influenced by the composition of growth medium where the $NH_4H_2PO_4$ and thiamine are the most influential factors ($p<0.05$). Optimum concentration of C1 and $NH_4H_2PO_4$ was 0.05% and 0.10% respectively and this is equivalent to 4.4 g/L and 8.2 g/L. The optimum concentration of thiamine and pyridoxine was 325 µg/L and 250 µg/L respectively. The optimum fermentation condition produced biomass of 7.57 g/L with biomass yield of 0.48 g biomass per g reducing sugar utilised.

Batch growth kinetics of *C. utilis* in a 2-L fermenter containing PWE, optimum PWE and YEPG medium were studied. Optimum PWE was significantly enhanced the biomass (11.73 g/L) compared to PWE (8.57 g/L) as the consequence of improved growth rate (0.96 h^{-1}). Saturation constant (*Ks*) and doubling time (t_D) were reduced while productivity (*P*) and yield over substrates ($Y_{x/s}$) were improved simultaneously. However, the oxygen demand (*SOUR*) of *C. utilis* in optimum PWE increased dramatically and oxygen became the limiting growth factor. Ability of *C. utilis* to utilise sucrose in PWE medium after a certain period of fermentation time was depicted by the electron micrographs showing deposition of endoplasmic reticulum along the periphery of cell membrane indicating enzyme synthesis which was not observed in YEPG medium.

1 INTRODUCTION

1.1 Background

Fermentation is the oldest and largest application of microbial technology. That involves the conversion of carbohydrates and related components to end products such as acids, alcohols and carbon dioxide (Bainotti *et al.*, 1996; Bamforth, 2005). Within the food industry, there are various types of organisms used in food fermentation. However, the principal organisms used are lactic acid bacteria and yeast such as baker's yeast which is commonly used as leavening agent in baking. Yeast is known as a good source of protein, amino acid, mineral such as phosphate and vitamin B. Single cell protein can serve as alternative protein source that alleviate the problem of protein scarcity (Anupama and Ravindra, 2000). While yeast extracts are also rich in amino acids, vitamins and trace minerals and often used as growth stimulants for microorganisms. Yeast extracts are also used widely as flavour improvers and enhancers to mask bitterness or sour taste and to increase aroma (Sommer, 1998). As the awareness of health issue surrounding the monosodium glutamate (MSG) as flavour enhancer increases, manufacturers seeking to replace MSG and achieve dramatic flavor enhancement through alternative sources. Hence, the demand of yeast extract is expanding.

In recent years, considerable research in converting agricultural waste, which is a renewable and abundantly available material, into value-added products have been carried out. For example, palm oil mill effluent (POME) and oil palm frond (OPF) (Loo *et al.*, 2002); pineapple waste (Nigam, 1999; Imandi *et al.*, 2008);

Chinese cabbage (Choi *et al.*, 2002); orange waste powder (Djekrif-Dakhmouche *et al.*, 2006); sugarcane bagasse (El-Nawwi and El-Kader, 1996) and rice straw hydrolysate (Zheng *et al.*, 2005) are amongst the agricultural wastes that have been successfully utilised as fermentation substrates. However, there are many agricultural wastes still underutilised.

Pineapple fruit is one of the popular fruits in Malaysia for processing and for fresh consumption either in the domestic or export markets. At present, the pineapple waste is not fully utilised. They are either used as animal feed or are discarded. However due to its high content of total sugar (approximately 83 g/L) and protein (6.40 g/L), pineapple waste can be used as a substrate for fermentation (Nigam, 1999). From a commercial perspective, recycling the waste would reduce the cost of waste disposal for canneries, as they are required to treat their waste before disposal in order to reduce the organic load to the environment (Imandi *et al.*, 2008).

Encouraging results were obtained from the feasibility studies using juice extracted from pineapple waste (PWE) as yeast fermentation medium. Based on the previous studies of Loo *et al.* (2002), pineapple waste produced the highest *Candida utilis* biomass, intracellular protein content and protease activity compared to the cultivation on POME and OPF. Whilst research by Liong (2003) found that, *C. utilis* grow better in PWE medium compared to *Saccharomyces cerevisiae*. Studies on optimum fermentation conditions (inoculum size, substrate ºBrix level, aeration rate and agitation speed) have also been carried out by Liong (2003) and Ooi (2006) on using pineapple wastes as cultivation medium of *C. utilis*.

Although pineapple wastes could be a good carbon source for yeast growth, the significantly lower nitrogen content of PWE (0.003-0.015%) as compared to the control defined medium of yeast extract-peptone-glucose (YEPG) which contains approximately 0.3% nitrogen content, resulted in the yeast biomass produced by PWE (3.16 g/L) to be lower compared to yeast biomass obtained from defined medium of YEPG (9.44 g/L) after 55 hours of fermentation (Liong, 2003). Thus, supplementation of nitrogen source is required to enhance the yeast growth as suggested by Ooi (2006). In addition, Mohd Azemi *et al.* (2001) reported that nitrogen supplementation was found to be essential for the growth of *C. utilis* in POME which has a much higher nitrogen content (0.033-0.037%).

In this project, optimum substrate concentration and inoculum size which had been determined by Liong (2003) were applied to further study the effects of different nitrogen sources and growth factors on *C. utilis* growth in shake flask fermentation. Optimum aeration rate and agitation speed which had been determined by Ooi (2006) were also applied in batch fermentation of *C. utilis* biomass with 2-L continuously stirred batch fermenter. Effects of nitrogen sources and growth factors were evaluated based on biomass of cell, productivity, yield and other growth kinetic parameters.

1.2 Objectives

The main objective of this research is to increase the biomass production of *C. utilis* cultivated in the pineapple waste medium by the addition nitrogen sources and growth factors. Thus, the specific objectives of this research work were:

a) to determine the effects of nitrogen sources (organic and inorganic) and growth factors on the yeast biomass production, yield and productivity.

b) to study the interaction effects among the selected factors and optimize the level of factors using response surface methodology (RSM) design.

c) to evaluate growth kinetic parameters and yeast cells morphological characteristic of *C. utilis* in different fermentation media.

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2 LITERATURE REVIEW

2.1 Agricultural wastes

Agriculture has played a vital role in the development of modern Malaysia and continues to be a major contributor to the Malaysian economy. Agriculture is designated to be the third engine of growth under the Eighth Malaysia Plan (2001–2005) (Agriculture in Malaysia, 2006). A strategic agricultural development master plan, The Third National Agricultural Policy (NAP3) was also formulated for years 1998–2010. This agro-food policy is directed towards increasing exports and reducing imports of agricultural commodities (Anonymous, 2004). **Table 2.1** showing the extrapolated agricultural production of the primary commodities in Malaysia for the year 2008 indicated an increase from the actual results in year 2005. It has resulted in the increase of agricultural land use from 5.7 million hectares to more than 6.0 million hectares in year 2008 (Crops statistic, 2008). However, the expansion of quantity of the land used for agriculture has increased the number of agriculture related environmental pollution. Traditional methods that were applied to manage crop residues through open-burning were prohibited as haze in South-East Asia reached critical levels (Ahmed *et al.*, 2004). Thus, one of the objectives of NAP3 is to conserve and utilise natural resources on a sustainable basis through developing industrial ecosystems where wastes from one economic activity are used as the inputs of other useful products. (Agriculture in Malaysia , 2006).

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Table 2.1: Agricultural production (metric tonnes) from 2000 – 2008. (Crops statistic, 2008)

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Commodity	2000	2005	2008[e]
Paddy	2,140,904	2,314,378	2,384,143
Fruits[1]	993,497	1,648,607	1,886,680
Pineapple[2]	-	355,937	323,747
Vegetable	404,671	580,738	816,244
Herbs	-	522	988
Spices	21,503	35,535	51,839
Flowers	120,353,234	135,145,712	16,595,290
Coconut	469,662	536,807	496,974
Coffee	77,241	35,408	28,685
Sugarcane	-	749,979	733,500
Tea	-	3,880	5,570

[e] Estimated production.
[1] Refers to commercial cultivation.
[2] Refers to subunit from fruits production

Based on the review by Anupama and Ravindra (2000), various agricultural wastes may serve as raw material that could be processed into protein-based value added products to relief the problem of protein scarcity, thus increasing the commercial value of agricultural production.

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Oil palm, as the largest agricultural product in Malaysia, contributes the largest share of total agricultural wastes which can be recycle and reuse. Despite the enormous amount of waste material produced by palm oil processing, the waste is to a large extent recyclable and reusable (Sharifuddin and Zaharah, 1989; Mohd Azemi *et al.*, 2001). The fronds and empty fruit bunches are reused in the plantation as mulching and to control soil erosion while other wastes are utilised as animal feed, organic fertilizer, fibreboard or as boiler fuel. During the replanting of oil palm, zero burning is now practiced as the felled trunks are recycled as organic fertilizer (Faridah, 2001).

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In Malaysia, agricultural wastes such as rice straws and husks, empty oil palm fruit bunches, saw dust, animal droppings, POME has been successfully recycled (Faridah, 2001). Bio-fuels are very desirable in view of serious concerns over the rising levels of greenhouse gases such as carbon dioxide, global warming and dwindling reserves of fossil fuels. Systematic programmes have been introduced to optimise the use of resources on a sustainable basis including the recycling the food production waste. Recycling of agricultural wastes also presents a great opportunity in supporting sustainable development by the production of bio-fuels and single cell proteins with yeast fermentation and, as reported by Nigam (1999) ethanol may also be obtained from pineapple cannery waste.

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2.1.1 Pineapple and utilisation of pineapple waste

Besides the agricultural wastes discussed in the previous section, much attention has been given to the utilisation of both fruit waste and vegetable processing industry wastes recently. Bioprocessing technologies involving utilisation of fruit and vegetable processing industry waste into the production of single cell protein and ethanol fermentation (Nigam, 1999; Tanaka *et al.*, 1999; Choi *et al.*, 2002); phenolic antioxidants (Correia *et al.*, 2004); citric acid (Imandi *et al.*, 2008) and other useful chemicals is beginning to get more attention.

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Pineapple waste is one of the potential wastes, as it is one of the main commodities in Malaysia for either domestic or export markets. Pineapple, *Ananas comosus* from Bromeliaceae family, originated from South America. It is a fruit rich in vitamins, fibres as well as many other nutrients and antioxidants (Morton, 1987).

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It can be found in plantations in Thailand, Philippines, Africa and other tropical countries. Over the past century, it has become one of the leading commercial fruit crops of the tropics. In Malaysia, pineapple plantations are found in Johore, Selangor, Kelantan and Penang with a total land use of 14,716 hectares and a yearly production of 323,747 million tons in year 2008 (Crops statistic, 2008). Malaysia exported most of its canned pineapple products to Europe between the 1960s to the 1990s. However, in the past decade, Malaysia's export of pineapple to Europe was declining while demands were increasing in East Asia, United States, Singapore and Japan. In 2006, fresh pineapple contributed RM 13.439 million of Malaysia's total export.

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In the process of canning the pineapple fruit, the outer peel and the central core are discarded. The waste, called pineapple bran, accounts for about 50% of the total pineapple weight, i.e. about 10 tons of fresh bran or one ton of dry bran per hectare. Pineapple bran, either fresh or dried, may be used as feed for ruminants and is usually combined with grass to form the roughage of the diet (Palafox and Reid, 1961). However, it is not an attractive use in animal feed as it contains, on a dry matter basis, high fibre content and soluble carbohydrates.

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Several studies have been carried out in Malaysia focused on pineapple solid and liquid waste, For example, a study on recycling the pineapple leaves to obtain sustainable potassium sources in land for agricultural use were carried out by Ahmed *et al.* (2004; 2005) while Norzita *et al.* (2005) obtained high yield of lactic acid production from both solid and liquid pineapple waste. Pineapple waste was also used in silage activities to ensure feed availability during periods of shortage (Chin, 2001). However, pineapple waste is still an underutilised agricultural waste in Malaysia and it is often used as fertilizer for other agricultural crops.

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Based on the chemical composition shown in **Table 2.2**, pineapple cannery waste is a potential source of sugars, protein, vitamins and other growth factors. Pineapple waste is favourable for yeast growth as reported by Nigam (1999) because it is able to yield large amounts of reducing sugars such as glucose and fructose that are most easily utilised by the yeast cell. Thus, pineapple cannery waste can be used as a substrate for ethanol production, and may reduce the costs of waste disposal in waste treatments before disposal in order to reduce the organic load. In addition, pineapple waste was used as substrate in solid substrate fermentation (SSF) for the prodcution of citric acid by *Yarrowia lipolytica* (Imandi *et al.*, 2008) or phenolic antioxidant by *Rhizopus oligosporus* (Correia *et al.*, 2004). However, pineapple contains relative low nitrogen content compared to the 0.5-1% (w/w) of nitrogen sources required for yeast growth (Lee and Kim, 2001).

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Table 2.2: Mean chemical composition of pineapple cannery waste[a] (Nigam, 1999)

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Chemical component	Concentration (g/L)
Total sugars	82.53 ± 0.78
Reducing sugars	39.46 ± 0.60
Glucose	22.70 ± 0.85
Sucrose	38.70 ± 1.12
Fructose	15.81 ± 0.83
Raffinose	2.62 ± 0.27
Galactose	2.85 ± 0.33
Protein	6.40 ± 0.33
Fat	1.20 ± 0.17
Kjeldahl nitrogen	2.32 ± 0.15
Total solids	50 – 60 *
Microbial count	$10^2 – 10^4$ mL^{-1} *
pH	4.0 ± 0.08

[a] Each value corresponds to the mean of five experiments ± SD (Standard Deviation).
* In the case of total solids and microbial count, SD is not estimated.

2.2 Yeast and *Candida utilis*

Yeasts are microscopic fungi. Its name is derived from the foam formed during fermentation. Yeasts were discovered only after the invention of the microscope as it is too minute to be observed by the naked eye. The basic shape of a yeast cell is taken to be a rotary ellipsoid with changes in the shape in two directions, either spherical shapes or elongated, and even filamentous (KocKová-Kratochvílová, 1990). It can also change shape during individual developmental stages. Yeasts propagate vegetatively by budding. The bud receives half of the vital structures of the original cell and grows up to nearly the same size so that both cells are about equivalent. Under low nutrient conditions, yeasts that are capable of sexual reproduction will form ascospores. Genus *Candida* is referred to those yeast that are not capable of going through the full sexual cycle, and hence, are also called imperfect fungi (Walker, 1998).

In yeast cytology, yeast biomass primarily comprises macromolecules (proteins, polysaccharides, lipids and nucleic acids) which are assembled into the structural components of the cell. Individual cells from a pure strain of a single species can also display morphological and colorimetric heterogeneity. However, profound effects in individual cell morphology are usually induced by alteration in physical and chemical conditions (Briggs *et al.*, 2004). A number of studies have demonstrated a correlation between cell morphology (especially cell wall structure) and natural sorption of cations in different yeast species. For example, Gniewosz *et al.* (2006) studied the capacity of *Saccharomyces cerevisiae* and *C. utilis* to bind magnesium. An essential difference was observed in the distribution of the total pool of magnesium bound with both strain of yeast cells. The cells of *S. cerevisiae* are

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characterized by a thicker cell wall, especially the outer glucan layer in comparison with the cells of *C. utilis*.

C. utilis, a unicellular eukaryote, belongs to the yeast family and is universally recognised as an important model for the study of genetics (Sengupta *et al.*, 1997). *C. utilis* is among the yeast, with a cell wall consisting primarily of hydrophobic polysaccharides. Its responses to external environmental conditions in changing cell wall composition and characteristic were explored and applied in many biosensors (Dhadwar *et al.*, 2003). Besides, *C. utilis* has been frequently used in biomass production because of its ability to utilise a variety of carbon sources and to support a high protein yield (Rajoka *et al.*, 2006). It also exhibits polyauxia which gradually utilise several substrates during fermentation. In term of enzymes, *C. utilis* may differ from other yeasts. For instance, it contains lipase which enables it to utilise hydrocarbon as carbon sources (KocKová-Kratochvílová, 1990).

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2.3 Basic nutrition for yeast

Yeast nutrition refers to the subsequent utilisation of essential food sources for both anabolic and catabolic reactions that ensure the growth and survival of the cell (Bamforth, 2005). Demands for satisfactory composition of the fermentation medium directly reflect the elementary composition of the biomass. Hence, addition of assimilable nitrogen sources and vitamins supplementation to yeast can improve its growth.

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2.3.1 Carbon sources

Yeasts are chemoheterotrophic organisms that require carbon and nitrogen, most often in the form of organic compounds. According to Cruz *et al.* (2002), the

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mutual interaction of carbon and nitrogen has an important role in the metabolism of living organisms. Carbohydrates are the most readily utilisable form of carbon in both oxidative and anoxidative way. Among the basic monosaccharides of hexoses, D-glucose, D-fructose and D-mannose which are used by all yeasts (KocKová-Kratochvílová, 1990), while sucrose is the dissaccharide most easily utilised by yeast cells as reported by Lee and Kim, (2001). However, it is noted that while glucose is routinely added to laboratory culture media for growing yeasts, glucose is not freely available in natural yeast habitats or in many industrial fermentation substrates (maltose, sucrose, fructose, xylose and lactose are the more common sugars). Indeed, glucose generally exhibits a repressive and inhibitory effect on the assimilation of other sugars by yeasts (Walker, 1998). Other saccharides, polyols, polysaccharides such as soluble starch, pectin; and alcohols such as ethanol, methanol, glycerol; as well as organic acids and other substances might be used by yeasts as carbon sources.

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Industrial cultivation of yeast biomass is carried out on various waste products that can de divided into traditional and non-traditional substrate. Saccharide substrates belong to the traditional waste substrates used for culturing of fodder yeast. Waste water for cultivation purposes, notably concentrated effluents of the food industry usually contains 2.5 – 7% utilisable saccharide and supplemented with minerals (nitrogen, phosphorus, potassium, magnesium, sulphur). It is noted that initial concentration of substrate will affect the production of the fermentation. Jinap *et al.* (1996) reported that initial concentration of glucose influenced the growth of *Hansenula anomala* and production of ester.

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There is increasing efforts being currently undertaken to discover non-traditional sources of yeast nutrition to obtain fodder protein from various waste materials (Ejiofor *et al.*, 1996; El-Nawwi and El-Kader, 1996; Arnold *et al.*, 2000). *C.*

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utilis is the primary yeast grown on these waste substrates (Sommer, 1998; Stabnikova *et al.*, 2005). Several species of the genus *Candida* are able to utilise D-xylose, L-arabinose, D-arabinose, L-sorbose, cellulose and trehalose. Although *C. utilis* has a relatively narrow range of sugars which can be considered as good growth and fermentation substrates; namely glucose, fructose and raffinose, *C. utilis* is able to assimilate some other sugars as shown in **Table 2.3**. Furthermore, the predominantly aerobic metabolism of *C. utilis* and active participation of the pentose phosphate pathway for sugar metabolism predisposes this yeast to carbon balance in favour of biomass production as compared with other yeasts such as *S. cerevisiae*, which are glucose sensitive and largely fermentative (Lee and Kim, 2001).

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Table 2.3: Properties of *Candida utilis* (KocKová-Kratochvílová, 1990)

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Property	Commodity
Fermentation	
Glucose	+
Galactose	-
Maltose	-
Sucrose	+
Lactose	-
Raffinose	+
Assimilation	
Glucose	+
Galactose	-
Maltose	+
Sucrose	+
Cellobiose	+
Trehalose	+
Lactose	-
Melibiose	-
Raffinose	+
Xylose	+
Starch	-
Salt concentration	6 to 8%
Maximum temperature	39 to 43%
Vitamin dependence	Thiamine, inositol and biotin or no vitamin dependence
KNO_3 assimilation	+

2.3.2 Nitrogen sources

Yeast cells have a nitrogen content of around 10% of their dry weight (KocKová-Kratochvílová, 1990). The source of nitrogen for yeasts is usually provided by organic compounds; some natural and seminatural media are based on peptone, yeast extract and others. Smith *et al.*, (1975) found that nitrogen is the main stimulatory factor in yeast extract as it encourages biostimulation on microbial growth. However, yeast extract in media contributes to a major cost in fermentation process. Minimum yeast extract supplementation or replacing yeast extract with a less expensive nitrogen source in order to develop an economically viable industrial process. The improvement of fermentation production has been studied under the control of various factors and media components (Arasaratnam *et al.*, 1996; Kim *et al.*, 2005) and industrial valorisations of agricultural subproduct like date juice, pineapple cannery effluent represents a useful research topic (Pessoa *et al.*, 1996; Nigam, 1999; Nancib *et al.*, 2001). Yeast extract consists primarily of amino acids to promote yeast growth (Chae *et al.*, 2001) that may not only serve as nitrogen but also carbon source. Cruz *et al.* (2002) suggested that when free amino acids are incorporated directly without modification into proteins or degraded by the cell; the nitrogen is then used for the synthesis of other nitrogenous cell constituents and the amino acid derivative keto-acids may be used by the cell for synthesis purposes.

Ammonium salts (sulphate, phosphate, nitrate) are common inexpensive nitrogen supplements used in fermentation (Rajoka *et al.*, 2006). Ammonium salts of organic acids are better utilised than salts from inorganic acids as the decomposition produces weak acids that can serve as an additional carbon source (Briggs *et al.*, 2004). Strong organic acids however, change the pH levels and have an inhibitory

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effect on cells. An exception is ammonium phosphate as phosphorus is a principal biogenic element and phosphoric acid acts a good buffer system.

Sims and Ferguson (1974) investigated the metabolism of ammonium ion (NH_4^+), suggested that 75% of NH_4^+ were incorporated into glutamate while the remainder into the amide group of glutamine. Glutamines or other amino acids can also be produced from glutamate via transamination. Therefore, glutamate is a central compound in the metabolism of nitrogen substances.

Candida is one of the strains that is able to assimilate nitrate ion (NO_3^-). Utilisation of potassium nitrate (KNO_3) is an important taxonomical feature as other genera including *Saccharomyces, Debaryomyces, Torulaspora, Pichia* are unable to utilise KNO_3 possibly due to the toxic nitrite produced during the reduction of nitrate (KocKová-Kratochvílová, 1990; Sengupta *et al.*, 1997).

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2.3.3 Phosphorus and sulphur

One of the important elements in nutrient media for yeasts is phosphorus. Phosphorus is crucial in the synthesis of substances such as phosphoproteins, phospholipids, nucleoproteins, nucleic acids, phophorylated polysaccharides and is also present in cells as inorganic orthophosphate, pyrophosphate, metaphosphate and polyphosphate (Walker, 1998; Briggs *et al.*, 2004). A significant contribution to the negative charge of the yeast cytoplasm is due to the presence of inorganic phosphates and the phosphate groups in organic compounds. The phosphate content of yeast cells constitutes around 3-5% of cell dry weight primarily in the form of orthophosphate (Aiking and Tempest, 1976; Theobald *et al.*, 1996). Phosphate is added to nutrient media in the form of potassium, ammonium or sometimes sodium

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phosphates. Molasses or other media with a low level of assimilatable nitrogen are usually supplied with ammonium phosphates.

Yeasts require sulphur principally for the biosynthesis of sulphur-containing amino acids. Yeast sulphur content represents around 0.3% of cell dry weight. Inorganic sulphate and the sulphur amino acid methionine are the two compounds central to the sulphur metabolism yeast. Methionine is the most effectively used amino acid in yeast nutrition (Walker, 1998).

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2.3.4 Growth factors

Growth factors constitute a diverse group of organic compounds required in very low concentrations for specific catalytic or structural roles in yeast but are not utilised as energy sources (Briggs *et al.*, 2004). Yeast growth factors include vitamins that serve as vital metabolic functions as components of coenzymes, purines and pyrimidines, nucleosides and nucleotides, amino acids, fatty acids, sterols and other miscellaneous compounds (e.g. polyamines, choline, meso-inositol) (Ahmad and Holland, 1995). For yeast cells, vitamins of the B group have been studied in considerable detail and yeast served as objects for elucidating their biogenesis (KocKová-Kratochvílová, 1990). However, according to Leopold and Španělově (1974), *C. utilis* does not require vitamins if the concentration of saccharides does not exceed 1% to 1.5%. The key vitamin requirements for yeast growth are biotin, pantothenic acid, pyridoxine, niacin and thiamine (Bamforth, 2005). Biotin must be present in the fermentation medium during processes used for the production of glutamic acid (Stanbury *et al.*, 1995). Content of growth factors in typical yeast and yeast extract are listed in **Table 2.4**.

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Table 2.4: Content (μg g^{-1}) of growth factors in yeast, yeast extract of *Candida utilis* and commercial yeast extract C1.

Growth factors (μg g-1)	Typical yeast	Typical yeast extract	Yeast extract of *C. utilis*	Yeast extract C1
m-Inositol	3000-5000	-	-	-
Pantothenate	80-150	-	-	-
Biotin	2-2.5	-	-	-
Thiamine	100-150	30	5.3	38.0
Pyridoxine	20-40	23	41.5	90.8
Niacin	400-600	680	664.2	665.0
Riboflavin	-	119	47.2	111.1
Folacin	-	-	-	-
Reference	Atkin (1949)	Sommer (1998)	Ooi (2006)	Liong (2003)

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2.3.5 Other basic nutrient of yeast

Water activity is an important factor affecting yeast growth. Yeasts are mostly mesophils and their growth intensity declines when the relative humidity drops below 98-97%. The concentration of available water elicits different responses in individual strains (Briggs *et al.*, 2004). However, some yeast strains can tolerate low water activity conditions; include *C. utilis*, *Pichia ohmeri, Hansenula anomola* (Rose and Harrison, 1995; Martorell *et al.*, 2007). A yeast cell requires oxygen only when it is in the form of molecular solution in water to serve in their oxidation processes. Oxygen depleted from the medium is replenished by diffusion from the atmosphere (KocKová-Kratochvílová, 1990). In addition, hydrogen ions (protons) are important in yeast cell physiology since variations in both extracellular and intracellular pH level can have a significant influence on growth and metabolism of yeast cells. Yeasts generally grow very well when the initial culture medium pH is between 4 - 6, but most yeast are also capable of growth over quite a wide range (Walker, 1998). However, Malakar *et al.* (2008) found that strong reagents like peroxide acid (H_2O_2), acetic acid, high sodium chloride (NaCl) or hydrochloric acid will induce apoptosis in the yeast.

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Konlani *et al.* (1996) reported that *C. krusei* and *Saccharomyces sp.* are a good source of mineral salts, particularly oligoelements, such as iron, magnesium, manganese, phosphorus and potassium. Hence, the addition of mineral elements in appropriate proportions to the nutrient medium would promote yeast proliferation. However, high quantities or unsuitable proportions may have a toxic effect especially micro or trace elements of mineral elements including manganese (Mn), calcium (Ca), iron (Fe), zinc (Zn), copper (Cu), nickel (Ni), cobalt (Co) and molybdenum (Mo). The addition of metal ions such as sodium in high concentrations exert a salt stress on yeast and induce apoptosis (Briggs *et al.*, 2004; Malakar *et al.*, 2008). Yeasts also have an absolute growth requirement for potassium which is essential as a cofactor for a wide variety of enzymes involved in oxidative phosphorylation, protein biosynthesis and carbohydrate metabolism.

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2.4 Yeast cell metabolism

Carbon and energy metabolism, assimilation and metabolism of essential inorganic nutrients such as nitrogen, phosphorus and sulphur are important in the process of understanding the yeast cell physiology.

2.4.1 Carbon and energy metabolism

Sugars are the preferred carbon and energy sources of most yeasts. The sequence of enzyme-catalysed reactions that oxidatively convert glucose to pyruvic acid in the yeast cytoplasm is known as glycolysis. Glycolysis provides yeast with energy, together with precursor molecules and reducing power for biosynthetic pathways (Briggs *et al.*, 2004). **Figure 2.1** shows that major sugar catabolic pathway of yeast cells during both aerobic and anaerobic respiration. For anaerobic

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fermentation, glycerol is the main product of yeast alcoholic fermentation, besides ethanol and carbon dioxide (Liu *et al.*, 2006). Other minor fermentation metabolites such as acetate esters groups are produced by yeast during the course of alcoholic fermentation (Van Iersel *et al.*, 1999). These products vary depending on the yeast strain and culture conditions.

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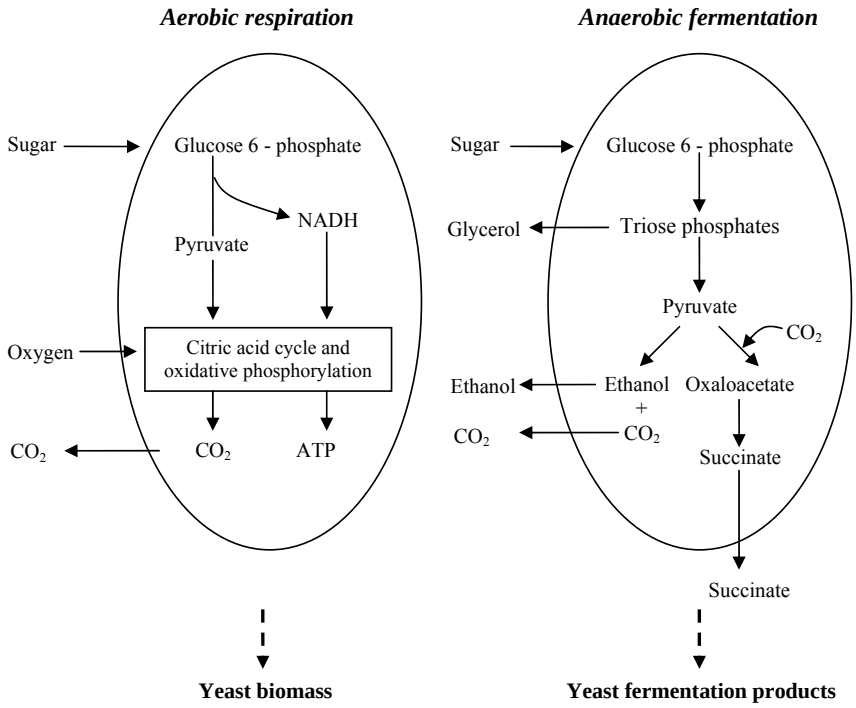

Figure 2.1: Summary of major sugar catabolic pathways in yeast cells (Walker, 1998)

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During the yeast respiration, glycolysis may be regarded as the prelude to the citric acid cycle (the Krebs cycle). It consists of electron transport chain and oxidative phosphorylation which collectively harvest most of the energy (in the form of ATP) from glucose. However, a greater array of carbon sources can be respired than fermented. Substrates which are respired by yeast cells include: pentose (e.g. xylose), sugar alcohols (e.g. glycerol), organic acids (e.g. acetic acid), aliphatic

alcohols (e.g. methanol, ethanol), hydrocarbons (e.g. *n*-alkanes) and aromatic compounds (e.g. phenol) (Sreekrishna *et al.*, 1997; Van Dijken *et al.*, 2000).

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The adaptability of yeasts to various growth environments and the presence of a particular regulatory phenomenon will very much depend on the prevailing growth conditions even within a single species. Crabtree effect relates glucose concentration with the particular catabolic route adopted by glucose-sensitive yeasts such as *S. cerevisiae*, even under aerobic conditions, fermentation predominated over respiration. But the Crabtree effect is not noticeable in glucose-insensitive yeasts (e.g. *C. utilis, Kluyveromyces marxianus*). *C. utilis*, a Crabtree-negative yeast, may limit its glycolytic rate by accumulating intracellular reserve carbohydrates or the cells may exhibit altered regulation of sugar uptake (Postma *et al.*, 1988; Van Dijken *et al.*, 2000). In batch culture, when the levels of consumed glucose decline, cells will gradually become depressed, resulting in an induction of respiratory enzyme synthesis. This, in turn, results in oxidative consumption of accumulated ethanol when cells enter a second phase of growth known as diauxie (Beudeker *et al.* 1989).

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2.4.2 Nitrogen metabolism

Yeasts are capable of utilising a range of nitrogen either inorganic or organic for incorporation into structural and functional nitrogenous components of the cell. In industrial fermentation media, available nitrogen is usually in the form of complex mixtures of amino acids, rather than ammonium salts. Nevertheless, media are often supplemented with inexpensive inorganic nitrogen, such as ammonium sulphates (Nancib *et al.*, 2001). Ammonium ions, is either supplied in nutrient media or derived from catabolism of other nitrogenous compounds, are actively transported and readily assimilated by all yeasts. For yeasts growing in the presence of

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ammonium salts, nitrogen is assimilated into glutamate and glutamine that serve as precursors for the biosynthesis of other amino acids. Since these amino acids derive their alpha-amino nitrogen directly from ammonia, they are synthesized at a rate sufficient to provide the alpha-amino nitrogen required for yeast cell growth. Other amino acids are formed by transamination reactions. Glutamate and glutamine are therefore primary products of ammonium assimilation and are key compounds in both nitrogen and carbon metabolism (Sengupta *et al.*, 1997; Briggs *et al.*, 2004; Kolkman *et al.*, 2006). **Figure 2.2** simplified the nitrogen assimilation in yeast cells.

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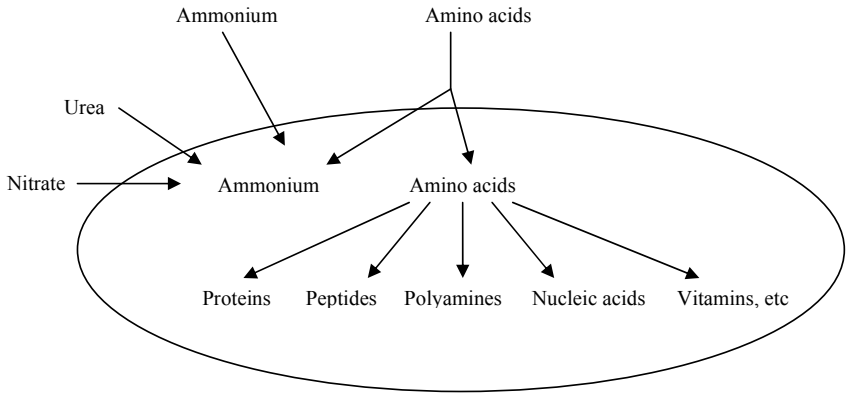

Figure 2.2: Overview of nitrogen assimilation in yeast cells (Walker, 1998)

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C. utilis is one of the yeast strains that able to transport and assimilate nitrate as a sole source of nitrogen. Assimilation into organic nitrogen is through the activities of nitrate reductase or nitrite reductase and formed ammonium ions (Sengupta *et al.*, 1997). Urea is widely used as inexpensive nitrogen in certain industrial fermentation feedstocks like molasses; depending on its extracellular concentration, it may enter cells by active transport or by facilitated diffusion (Walker, 1998). However, urea would not be recommended as a nutritional

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supplement in fermentations for potable spirit beverage production due to the possible formation of carcinogenic ethylcarbamate which is formed as a reaction product between ethanol and residual urea during the distillation process (Beudeker *et al.* 1989).

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2.4.3 Phosphorus and sulphur metabolism

Phosphorus requirements of yeast cells are met by the uptake of inorganic phosphate ions from growth media. The phosphate taken up will eventually be incorporated into major cell constituents (phospholipids, nucleic acids, proteins) or may be employed in the numerous transphosphorylation reactions of intermediary metabolism. Phosphate transport into the cell is dependent on energy metabolism and is primarily regulated by the intracellular orthophosphate concentration (KocKová-Kratochvílová, 1990). The sulphur requirements of almost all yeasts can be met through assimilatory sulphate reduction and subsequent incorporation into sulphur amino acids. Briggs *et al.* (2004) suggested that carbohydrate metabolism may influence the sulphur metabolism. Many yeasts can grow on sulphite or methionine while only some of them, notably *C. utilis*, can grow on cysteine and cystine. (Walker, 1998).

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2.5 Growth kinetics in yeasts

2.5.1 Cell cycle of yeast

The cell cycle can be defined as the period between division of a mother cell and subsequent division of its daughter progeny. Growth is rate-limiting for cell cycle progress, which is modulated by physiological factors such as nutrient availability. The course of culture growth can be assessed by determining the

increase in the number of cells at regular time intervals after inoculation (Stanbury *et al.*, 1995). An S-shaped growth curve is usually obtained among batch growths when the logarithm of the number of cells is plotted against time unit (**Figure 2.3**) demonstrating that the culture passes through several phases of the growth cycle during its growth, generally comprised of lag, exponential and stationary phases (Walker, 1998).

The lag phase represents a period of zero growth (specific growth rate, μ=0) and is exhibited when inoculum cells experience a change of nutritional status or alterations in physical growth conditions (e.g. temperature, osmolarity). The precise duration of the lag phase is dependent on inoculation density (Briggs *et al.*, 2004). A cell transferred to a new nutrient medium has to adapt to the changed conditions and must take up nutrients to create an energy reserve for future multiplication (Stanbury *et al.*, 1995). Hence, the lag phase reflects the time required for inoculated yeast cells to adapt by synthesizing ribosomes and enzymes needed to establish growth at a higher rate. At the end of this stage, the cell usually assumes its most elongated shape.

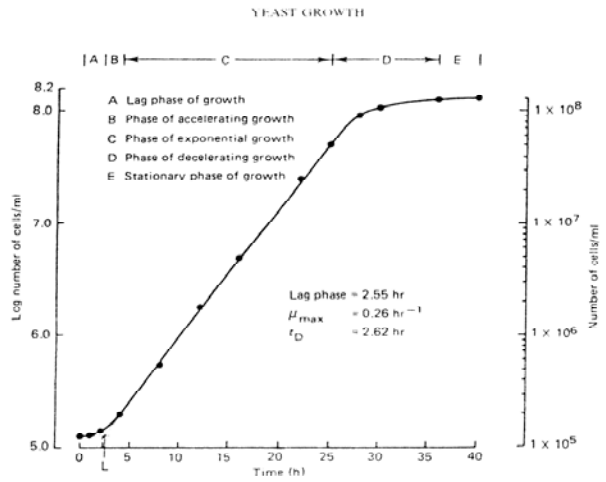

Figure 2.3: Typical yeast growth curve. The figure shows the phases of growth of yeast in batch culture. μ_{max} is the maximum specific growth rate and t_D is the doubling time. Reproduced from Hough (1985) as cited by Walker (1998).

When abundant multiplication takes place (exponential phase), the cells separate from the mother cell before attaining their normal dimensions. When the multiplication gradually ceases the cells begin to grow to attain a larger size during stationary phase. Upon maturity, their shape and size become the same as those of the mother cell.

Once cells transit from the lag phase and commence active cell division, they enter an acceleration phase before exponential growth. The rate increase (dx/dt) in yeast biomass (x) with time (t) during this phase is expressed as

$$\frac{dx}{dt} = \mu x$$

with the value of the specific growth rate (μ) varying between 0 (lag phase) and μ_{max} (Ahmad and Holland, 1995; Stanbury *et al.*, 1995).

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The exponential phase represents a period of logarithmic cell doublings and constant, maximum specific growth (μ_{max}). The precise value of μ_{max} (in dimension of reciprocal time, h^{-1}) depends on the yeast species and the prevailing growth conditions. If growth is optimal, the cells double logarithmically (Demirtas *et al.*, 2003), then

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$$\frac{dx}{dt} = \mu_{max} x$$

when integrated, this yields

$$\ln x - \ln x_0 = \mu_{max} t$$

(where x_0 is the initial cell mass) or

$$x = x_0 e^{(\mu_{max} t)}$$

The doubling time (t_D) of a culture from knowledge of μ_{max} can be calculated as

$$t_D = \frac{\ln 2}{\mu_{max}} = \frac{0.693}{\mu_{max}}$$